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<b>(21) International Application Number:</b> PCT/IB97/00404 <b>(22) International Filing Date:</b> 1 April 1997 (01.04.97)  <b>(30) Priority Data:</b> 196 12 967.2      1 April 1996 (01.04.96)      DE  <b>(71) Applicant (for all designated States except US):</b> CHIRON BEHRING GMBH & CO. [DE/DE]; Postfach 1630, D- 35006 Marburg (DE).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> GRÖNER, Albrecht [DE/DE]; Fasanenweg 6, D-64342 Seeheim (DE).  <b>(74) Agent:</b> HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> PROCESSES FOR THE REPLICATION OF INFLUENZA VIRUSES IN CELL CULTURE, AND THE INFLUENZA VIRUSES OBTAINABLE BY THE PROCESS  <b>(57) Abstract</b>  Novel processes for the replication of influenza viruses in cell culture, and vaccines and diagnostic compositions which contain the influenza viruses obtainable by the process or constituents thereof, are described.		

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5   Processes for the replication of influenza viruses in  
cell culture, and the influenza viruses obtainable by  
the process

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          The present invention relates to processes for  
the replication of influenza viruses in cell culture at  
reduced temperatures, and to the influenza viruses  
obtainable by the process described and to vaccines  
15   which contain viruses of this type or constituents  
thereof.

          All influenza vaccines which have been used  
since the 40s until today as permitted vaccines for the  
treatment of humans and animals consist of one or more  
20   virus strains which have been replicated in embryonate  
hens' eggs. These viruses are isolated from the  
allantoic fluid of infected hens' eggs and their  
antigens are used as vaccine either as intact virus  
particles or as virus particles disintegrated by  
25   detergents and/or solvents - so-called cleaved  
vaccine - or as isolated, defined virus proteins - so-  
called subunit vaccine. In all permitted vaccines, the  
viruses are inactivated by processes known to the  
person skilled in the art. Even the replication of live  
30   attenuated viruses, which are tested in experimental  
vaccines, is carried out in embryonate hens' eggs.  
The use of embryonate hens' eggs for vaccine production  
is time-, labor- and cost-intensive. The eggs - from  
healthy flocks of hens monitored by veterinarians -  
35   have to be incubated before infection, customarily for  
12 days. Before infection, the eggs have to be selected  
with respect to living embryos, as only these eggs are  
suitable for virus replication. After infection the  
eggs are again incubated, customarily for 2 to 3 days.

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The embryos still alive at this time are killed by cold and the allantoic fluid is then obtained from the individual eggs by aspiration. By means of laborious purification processes, substances from the hen's egg which lead to undesired side effects of the vaccine are separated from the viruses, and the viruses are concentrated. As eggs are not sterile (pathogen-free), it is additionally necessary to remove and/or to inactivate pyrogens and all pathogens which are possibly present. To increase the virus yield, the replication of the influenza viruses in hens' eggs as a rule is carried out at reduced temperatures (about 34°C). Even viruses which cause respiratory diseases can be replicated in cell culture. Here too, in some cases reduced temperatures are used (about 33°C), which, however, have no effect on the quality of a vaccine which may be obtained, but only favor replication.

Viruses of other vaccines such as, for example, rabies viruses, mumps, measles and rubella viruses, polio viruses and FSME viruses can be replicated in cell cultures. As cell cultures originating from tested cell banks are pathogen-free and, in contrast to hens' eggs, are a defined virus replication system which (theoretically) is available in almost unlimited amounts, they make possible economical virus replication under certain circumstances even in the case of influenza viruses. Economical vaccine production is possibly also achieved in that virus isolation and purification from a defined, sterile cell culture medium appears simpler than from the strongly protein-containing allantoic fluid.

The isolation and replication of influenza viruses in eggs leads to a selection of certain phenotypes, of which the majority differ from the clinical isolate. In contrast to this is the isolation and replication of the viruses in cell culture, in which no passage-dependent selection occurs (Oxford, J.S. et al., J. Gen. Virology 72 (1991), 185 - 189; Robertson, J.S.

et al., J. Gen. Virology 74 (1993) 2047 - 2051). For an effective vaccine, therefore, virus replication in cell culture is also to be preferred from this aspect to that in eggs. It is known that influenza viruses can be replicated in cell cultures. Beside hens' embryo cells and hamster cells (BHK21-F and HKCC), MDBK cells, and in particular MDCK cells have been described as suitable cells for the in-vitro replication of influenza viruses (Kilbourne, E. D., in: Influenza, pages 89 - 110, Plenum Medical Book Company - New York and London, 1987). A prerequisite for a successful infection is the addition of proteases to the infection medium, preferably trypsin or similar serine proteases, as these proteases extracellularly cleave the precursor protein of hemagglutinin [HA<sub>0</sub>] into active hemagglutinin [HA<sub>1</sub> and HA<sub>2</sub>]. Only cleaved hemagglutinin leads to the adsorption of the influenza viruses on cells with subsequent virus assimilation into the cell (Tobita, K. et al., Med. Microbiol. Immunol., 162 (1975), 9 - 14; Lazarowitz, S.G. & Choppin, P.W., Virology, 68 (1975) 440 - 454; Klenk, H.-D. et al., Virology 68 (1975) 426 - 439) and thus to a further replication cycle of the virus in the cell culture.

The Patent US 4 500 513 described the replication of influenza viruses in cell cultures of adherently growing cells. After cell proliferation, the nutrient medium is removed and fresh nutrient medium is added to the cells with infection of the cells with influenza viruses taking place simultaneously or shortly thereafter. A given time after the infection, protease (e.g. trypsin) is added in order to obtain an optimum virus replication. The viruses are harvested, purified and processed to give inactivated or attenuated vaccine. Economical influenza-virus replication as a prerequisite for vaccine production cannot be accomplished, however, using the methodology described in the patent mentioned, as the change of media, the subsequent infection as well as the addition of trypsin which is carried out later necessitate opening the

individual cell-culture vessels several times and is thus very labor-intensive. Furthermore, the danger increases of contamination of the cell culture by undesirable microorganisms and viruses with each  
5 manipulation of the culture vessels. A more cost-effective alternative is cell proliferation in fermenter systems known to the person skilled in the art, the cells growing adherently on microcarriers. The serum necessary for the growth of the cells on the  
10 microcarriers (customarily fetal calf serum), however, contains trypsin inhibitors, so that even in this production method a change of medium to serum-free medium is necessary in order to achieve the cleavage of the influenza hemagglutinin by trypsin and thus an  
15 adequately high virus replication. Thus this methodology also requires opening of the culture vessels several times and thus brings with it the increased danger of contamination.

The present invention is thus based on the  
20 object of making available processes which make possible simple and economical influenza virus replication in cell culture and lead to a highly efficacious vaccine.

This object is achieved by the provision of the  
25 embodiments indicated in the patent claims.

The invention thus relates to a process for the replication of influenza viruses in cell culture, in which cells which can be infected by influenza viruses are cultured in cell culture, the cells are infected  
30 with influenza viruses and after infection are cultured at a temperature in the range from 30 to 36°C for virus replication.

In a preferred embodiment of the process according to the invention, the culturing of the  
35 infected cells for virus replication is carried out at 32 to 34°C and particularly preferably at 33°C.

It has surprisingly been found that by the replication of the influenza viruses in infected cells at reduced temperatures, viruses are obtained which

have an appreciably higher efficacy as vaccine than those viruses which are obtained by replication at 37°C. Replication at 37°C, the customarily used temperature for influenza replication in cell culture, admittedly leads to comparatively high virus yields in a short time. However, the viruses thus produced have a low efficacy as vaccine in comparison with viruses which are prepared by the process according to the invention.

The cells which are used in the process according to the invention for replication of the influenza viruses can in principle be any desired type of cells which can be cultured in cell culture and which can be infected by influenza viruses. They can be both adherently growing cells or else cells growing in suspension.

In a preferred embodiment, the cells are vertebrate cells, in particular avian cells and in this context preferably hens' cells, for example hens' embryo cells (CEF cells).

In a further preferred embodiment, the cells are mammalian cells, for example hamster, cattle, monkey or dog cells. Preferably, kidney cells or cell lines derived from these are used. Examples of suitable hamster cells are the cell lines having the names BHK21-F or HKCC. Possible monkey cells are, for example, VERO cells, and possible cattle cells are the MDBK cell line. An example of a suitable kidney cell line is the cell line MDCK (ATCC CCL34 MDCK (NBL-2)) from dog kidneys.

In the context of the present invention, a further cell line was established from the abovementioned kidney cell line MDCK, which further cell line is adapted to growth in suspension in serum-free medium and thereby makes possible particularly simple and efficient culturing and virus replication. This cell line, MDCK 33016, is particularly preferably used in the process according to the invention. It was deposited under the deposit number DSM ACC 2219 on

June 7, 1995 according to the requirements of the Budapest Convention on the Recognition of the Deposition of Microorganisms for the purposes of patenting in the German Collection of Microorganisms  
5 (DSM) in Brunswick (Federal Republic of Germany), which is recognized as the international deposition site.

For culturing the cells in the process according to the invention, the customary methods known to the person skilled in the art can be used for cell  
10 culture, in particular those which are already known for the replication of influenza viruses in cell culture. The carrying-out of the process according to the invention using cells which grow in-suspension, in particular those which can be cultured in serum-free  
15 medium, makes possible particularly simple and efficient virus replication. Culturing of the cells in suspension can in this case be carried out both in the batch process and in the perfusion system, e.g. in a stirred vessel fermenter, using the cell retention  
20 systems known to the person skilled in the art, such as, for example, centrifugation, filtration, spin filters and the like.

The culturing of the cells is carried out as a rule at a regulated pH which is preferably in the range  
25 from pH 6.6 to pH 7.8, in particular in the range from pH 6.8 to pH 7.3.

Furthermore, the  $pO_2$  value can advantageously be regulated and is then as a rule between 25% and 95%, in particular between 35% and 60% (based on the air  
30 saturation).

The infection of the cells cultured in suspension is preferably carried out when the cells in the batch process have reached a cell density of about  $8$  to  $25 \times 10^5$  cells/ml or about  $5$  to  $20 \times 10^6$  cells/ml in the  
35 perfusion system. If adherently growing cells are used, the optimum cell density for infection depends on the particular cell line.

The infection of the cells with influenza viruses is preferably carried out at an m.o.i. (multiplicity of



infection) of about 0.0001 to 10, preferably of 0.002 to 0.5.

5 The addition of a protease which brings about the cleavage of the precursor protein of hemagglutinin [HA<sub>0</sub>] and thus the adsorption of the viruses to the cells, can be carried out according to the invention shortly before, simultaneously with or shortly after the infection of the cells with influenza viruses. If the addition is carried out simultaneously with the infection, the protease can either be added directly to 10 the cell culture to be infected or, for example, as a concentrate together with the virus inoculate. If a serum-containing medium is used for culturing, this should be removed before protease addition. The protease is preferably a serine protease, and particularly preferably trypsin. 15

If trypsin is used, the final concentration added in the culture medium is advantageously 1 to 200 µg/ml, preferably 5 to 50 µg/ml, and particularly 20 preferably 5 to 30 µg/ml.

After infection, the infected cell culture is cultured further to replicate the viruses, in particular until a maximum cytopathic effect or a maximum amount of virus antigen can be detected.

25 In a preferred embodiment of the process, the harvesting and isolation of the replicated influenza viruses is carried out 2 to 10 days, preferably 3 to 7 days, after infection. To do this, for example, the cells or cell residues are separated from the culture medium by means of methods known to the person skilled 30 in the art, for example by separators or filters. Following this the concentration of the influenza viruses present in the culture medium is carried out by methods known to the person skilled in the art, such as, for example, gradient centrifugation, filtration, 35 precipitation and the like.

The invention further relates to influenza viruses which are obtainable by a process according to the invention. These can be formulated by known methods

to give a vaccine for administration to humans or animals. As already explained above, influenza viruses of this type have a higher efficacy as vaccine than influenza viruses which are obtained by replication at  
5 37°C in cell culture.

The immunogenicity or efficacy of the influenza viruses obtained as vaccine can be determined by methods known to the person skilled in the art, e.g. by means of the protection imparted in the exposure experiment or as  
10 antibody titers of virus-neutralizing antibodies.

The determination of the amount of virus or antigen produced can be carried out, for example, by the determination of the amount of hemagglutinin by methods known to the person skilled in the art. It is known,  
15 for example, that cleaved hemagglutinin binds to erythrocytes of various species, e.g. to hens' erythrocytes. This makes possible a simple and rapid quantification of the viruses produced or of the antigen formed by appropriate detection methods.

20 By means of comparison experiments in animal models, it was demonstrated that influenza viruses according to the invention produce an appreciably higher titer of neutralizing antibodies than viruses replicated at 37°C and thereby impart an appreciably  
25 better protection against influenza virus infection. In experiments with mice as an animal model, the titer of neutralizing antibodies was, for example, higher by at least a factor of 42 weeks after vaccination than the titer of neutralizing antibodies after inoculation with  
30 influenza viruses which had been replicated at 37°C. 4 weeks after the inoculation, the titer of neutralizing antibodies was higher by at least a factor of 17 and in some cases up to 27 times higher. If a revaccination was carried out, the titer of neutralizing antibodies  
35 could be higher by a factor of over 60 when using influenza viruses according to the invention in comparison with influenza viruses which had been replicated at 37°C. Accordingly, the survival rate of animals in an exposure experiment using an

administration of 1000 LD<sub>50</sub> (lethal dose 50%) can be increased from 1/10 to at least 8/10, preferably to 9/10 and particularly preferably to 10/10 (100%).

The invention further relates to vaccines which  
5 contain influenza viruses obtainable from the process according to the invention. Vaccines of this type can optionally contain the additives customary for vaccines, in particular substances which increase the immune response, i.e. so-called adjuvants, e.g.  
10 hydroxides of various metals, constituents of bacterial cell walls, oils or saponins, and moreover customary pharmaceutically tolerable excipients.

The viruses can be present in the vaccines as intact virus particles, in particular as live  
15 attenuated viruses. For this purpose, virus concentrates are adjusted to the desired titer and either lyophilized or stabilized in liquid form.

In a further preferred embodiment, the vaccines according to the invention can contain disintegrated,  
20 i.e. inactivated, or intact, but inactivated viruses. For this purpose, the infectiousness of the viruses is destroyed by means of chemical and/or physical methods (e.g. by detergents or formaldehyde). The vaccine is then adjusted to the desired amount of antigen and  
25 after possible admixture of adjuvants or after possible vaccine formulation, dispensed, for example, as liposomes, microspheres or slow release formulations.

In a further preferred embodiment, the vaccine according to the invention can finally be present as  
30 subunit vaccine, i.e. it can contain defined, isolated virus constituents, preferably isolated proteins of the influenza virus. These constituents can be isolated from the influenza viruses by methods known to the person skilled in the art.

35 The difference that the influenza viruses according to the invention, which were prepared at lower temperatures, have a higher antigenicity than viruses which were prepared according to conventional methods as higher temperatures, can be used for

diagnostic purposes. Therefore the present invention also relates to diagnostic compositions which contain influenza viruses according to the invention or constituents of such viruses, if appropriate in combination with additives customary in this field and suitable detection agents.

The examples illustrate the invention.

#### 10 Example 1

##### Replication of influenza viruses in MDCK-cells at 33°C

15 MDCK cells (ATCC CCL 34) were replicated in cell culture bottles (Eagle's MEM [EMEM] using 2% FCS, incubation at 37°C for 4 days). The resulting dense cell lawn was detached from the vessel wall using trypsin solution, the cells were isolated and the cell  
20 concentrate was resuspended in serum-containing medium. The cells were inoculated into roller bottles (200 ml/bottle) at a cell density of  $5 \times 10^5$  cells/ml and incubated at 37°C at 4 rpm. After 2 days, the cells were infected with influenza viruses. To do this, the  
25 medium above the dense cell lawn was removed and replaced by serum-free EMEM. Influenza virus A/PR/8/34 with an m.o.i. (multiplicity of infection) of 0.1 and trypsin in a final concentration of 25 µg/ml were added to the medium. Two roller bottles in each case were  
30 incubated at 37°C or at 33°C. The virus replication was determined as amount of antigen (measured as hemagglutinin units) and as infectiousness (measured in the CC ID<sub>50</sub> test) was determined and is shown in Table 1.

Table 1

Replication of influenza A/PR/8/34 in roller bottles (MDCK cell line) after incubation at 37°C and 33°C, measured as antigen content (HA units and infectiousness) (CCID<sub>50</sub>)

	HA content			CCID <sub>50</sub> /ml [log <sub>10</sub> ]
	2 dpi	3 dpi	4 dpi	4 dpi
37°C	1:128	1:512	1:1024	6.4
33°C	1:64	1:256	1:1024	5.7

dpi = days after infection

10

The ratios indicated mean that a 1:X dilution of the virus harvest still has hemagglutinating properties. The hemagglutinating properties can be determined, for example, as described in Mayer et al., Virologische Arbeitsmethoden, [Virological Working Methods, Volume 1 (1974), pages 260-261 or in Grist, Diagnostic Methods in Clinical Virology, pages 72-75.

15

The determination of the CCID<sub>50</sub> value can be carried out, for example, according to the method which is described in Paul, Zell- und Gewebekultur [Cell and tissue culture] (1980), p. 395.

20

#### Example 2

25

Preparation of a cell line which is adapted to growth in suspension and can be infected by influenza viruses

A cell line which is suited to growth in suspension culture and can be infected by influenza viruses was selected starting from MDCK cells (ATCC CCL34 MDCK (NBL-2), which had been proliferated by means of only a few passages or over several months in the laboratory. This selection was carried out by

30

proliferation of the cells in roller bottles which were rotated at 16 rpm (instead of about 3 rpm as customary for roller bottles having adherently growing cells). After several passages of the cells present suspended  
5 in the medium, cell strains growing in suspension were obtained. These cell strains were infected with influenza viruses and the strains were selected which produced the highest virus yield. An increase in the rate of cells growing in suspension during the first  
10 passages at 16 rpm is achieved over 1 to 3 passages by the addition of selection systems known to the person skilled in the art, such as hypoxanthine, aminopterin and thymidine, or alanosine and adenine, individually or in combination. The selection of cells growing in  
15 suspension is also possible in other agitated cell culture systems known to the person skilled in the art, such as stirred flasks. An example of cells which are adapted to growth in suspension and can be infected by influenza viruses is the cell line MDCK 33016  
20 (DSM ACC2219).

### Example 3

Replication of influenza viruses in MDCK 33016 cells at  
25 33°C

The cell line MDCK 33016 (DSM ACC2219) growing in suspension was replicated at 37°C in Iscove's medium with a splitting rate of 1:8 to 1:12 twice weekly in a  
30 roller bottle which rotated at 16 rpm. 4 days after transfer, a cell count of approximately  $7.0 \times 10^5$  cells/ml was achieved. Simultaneously with the infection of the now 4-day old cell culture with the influenza strain A/PR/8/34 (m.o.i. = 0.1), the cell  
35 culture was treated with trypsin (25 µg/ml final concentration), incubated further at 37°C or 33°C and the virus replication was determined over 3 days (Tab. II).

Table II

Replication of influenza A/PR/8/34, measured as antigen content (HA units) in roller bottles (MDCK cell line MDCK 33016) after infection of a cell culture without change of medium at an incubation temperature of 37°C or 33°C

	HA content after days after infection (dpi)		
	1 dpi	2 dpi	3 dpi
37°C	1:64	1:512	1:1024
33°C	1:16	1:128	1:1024

#### 10 Example 4

Replication of various influenza strains in MDCK 33016 cells (DSM ACC 2219) at 33°C

15 The cell line MDCK 33016 (DSM ACC 2219) was proliferated at 37°C in Iscove's medium with a splitting rate of 1:8 to 1:12 twice weekly in a roller bottle which rotated at 16 rpm. 4 days after transfer, a cell count of approximately  $7.0 \times 10^5$  to  $10 \times 10^5$  cells/ml was achieved. Simultaneously with the infection of the now 4-day old cell culture with various influenza strains (m.o.i.  $\approx 0.1$ ), the cell culture was treated with trypsin (25  $\mu\text{g/ml}$  final concentration) and incubated further at 33°C, and the virus replication was determined on the 5th day after infection (Table III).

Table III

Replication of influenza strains in roller bottles  
(cell line MDCK 33016) after infection of a cell  
5 culture without change of medium, measured as antigen  
content (HA units)

Influenza strain	HA content 5 days after infection HA content
A/Singapore	1:1024
A/Sichuan	1:256
A/Shanghai	1:256
A/Guizhou	1:128
A/Beijing	1:512
B/Beijing	1:256
B/Yamagata	1:512
A/PR/8/34	1:1024
A/Equi 1/Prague	1:512
A/Equi 2/Miami	1:256
A/Equi 2 Fontainebleau	1:128
A/Swine/Ghent	1:512
A/Swine/Iowa	1:1024
A/Swine/Arnsberg	1:512

**Example 5**

10

**Preparation of an experimental influenza vaccine**

After inoculation in mice, human-pathogenic  
influenza viruses customarily do not lead to their  
15 infection with pathological processes, so that  
protection experiments with mice are experimentally  
very difficult to construct. The influenza virus strain  
A/PR/8/34, however, is adapted to mice and after



intranasal administration causes a dose-dependent mortality in mice.

5 An experimental vaccine was prepared from influenza virus A/PR/8/34 from Example 3 (A/PR/8 replicated at 37°C or 33°C). The influenza viruses in cell culture medium were separated from cells and cell fragments by low-speed centrifugation (2000 g, 20 min, 4°C) and purified by a sucrose gradient centrifugation (10 to 50% (wt/wt) of linear sucrose gradient, 10 30,000 g, 2 h, 4°C). The influenza virus-containing band was obtained, diluted with PBS pH 7.2 1:10, and sedimented at 20,000 rpm, and the precipitate was taken up in PBS (volume: 50% of the original cell culture medium). The influenza viruses were inactivated with 15 formaldehyde (addition twice of 0.025% of a 35% strength formaldehyde solution at an interval of 24 h, incubation at 20°C with stirring).

10 NMRI mice each, 18 to 20 g in weight, were inoculated with 0.3 ml each of these inactivated 20 experimental vaccines on day 0 and day 28 by subcutaneous injection. 2 and 4 weeks after the inoculation and also 1 and 2 weeks after revaccination, blood was taken from the animals to determine the titer of neutralizing antibodies against A/PR/8/34. To 25 determine the protection rate, the mice were exposed 2 weeks after revaccination (6 weeks after the start of the experiment) by intranasal administration of 1000 LD<sub>50</sub> (lethal dose 50%). The results of the experiment are compiled in Table IV.

Table IV

Efficacy of experimental vaccines: for vaccine A the influenza virus A/PR/8/34 was replicated at 37°C and for vaccine B at 33°C. The titers of neutralizing antibodies against A/PR/8 and also the protection rate after exposure of the mice were investigated.

Titer of neutralizing antibodies/ml*					Protection rate
					Number
					living/total
	2 w pvacc	4 w pvacc	1 w prevacc	2 w prevacc	
37°C	<28	56	676	1 620	1/10
33°C	112	1 549	44 670	112 200	9/10

\* Weeks after vaccination (w pvacc) and weeks after revaccination (w prevacc)

The experiments confirm that influenza viruses which had been replicated at 37°C in cell culture with a high antigen yield (HA titer) only induced low neutralizing antibody titers in the mouse and barely provided protection, while influenza viruses which had been replicated at 33°C in cell culture also with a high antigen yield (HA titer) induced very high neutralizing antibody titers in the mouse and led to very good protection.

#### Example 6

Replication of influenza viruses in MDCK cells at 33°C and efficacy of the vaccine obtained

The cell line MDCK (ATCC CL34) was replicated at 37°C in a cell culture bottle in Eagle's MEM (EMEM) with 2% FCS with a splitting rate of 1:8 to 1:12 twice weekly. 4 days after transformation, a dense cell lawn had resulted. After change of the medium to serum-free EMEM, the cell culture was infected with influenza

B/Beijing (m.o.i. = 0.1), trypsin was added to the medium in a final concentration of 25 µg/ml and the infected cell culture bottles were incubated either at 37°C or at 33°C. 4 days after infection, the HA content in both experimental batches was 256 HA units. After low-speed centrifugation to remove cells/cell residues, the viruses in the supernatant were inactivated with formaldehyde (addition two times of 0.025% of a 35% strength formaldehyde solution at an interval of 24 h, incubation at 20°C with stirring). In each experimental section, the adjuvant added was aluminum hydroxide (10% final concentration of a 2% strength Al(OH)<sub>3</sub> solution). Using these experimental vaccines, in each case 3 guinea-pigs (400 to 500 g) per experimental section underwent intraplantar vaccination with 0.2 ml and revaccination 4 weeks afterwards with the same vaccine. To investigate the efficacy of the vaccine, blood samples were taken 2, 4 and 6 weeks after inoculation and tested in the hemagglutination inhibition test and serum neutralization test (cf. Table V).

Table V

Efficacy of experimental vaccines from influenza B/Beijing after replication of the virus at 37°C and 33°C in cell culture: the serological parameters hemagglutination inhibition and neutralizing antibodies were investigated (average values of 3 guinea-pigs)

Hemagglutination inhibition			Neutralizing antibodies			
	Titer			Titer		
	2 w	4 w	6 w	2 w	4 w	6 w
	pvacc*	pvacc*	pvacc	pvacc	pvacc	pvacc
37°C	85	341	1024	851	1290	6760
33°C	85	341	853	3890	22400	117490

\* w pvacc = weeks after inoculation (6 w pvacc = 2 weeks after revaccination)

**Patent claims:**

- 5 1. A process for the replication of influenza viruses in cell culture, in which cells which can be infected by influenza viruses are cultured in cell culture, the cells are infected with influenza viruses and after infection are cultured at a temperature in  
10 the range from 30° to 36°C for virus replication.
2. The process as claimed in claim 1, in which the cells are cultured with influenza viruses at a temperature in the range from 32°C to 34°C after infection for virus replication.
- 15 3. The process as claimed in claim 2, in which the cells are cultured with influenza viruses at 33°C after infection for virus replication.
4. The process as claimed in one of claims 2 and 3, in which the cells are vertebrate cells.
- 20 5. The process as claimed in claim 4, in which the vertebrate cells are avian cells.
6. The process as claimed in claim 5, in which the cells are hens' embryo cells.
7. The process as claimed in claim 4, in which the  
25 vertebrate cells are mammalian cells.
8. The process as claimed in claim 7, in which the mammalian cells are hamster, cattle, monkey or dog cells.
9. The process as claimed in one of claims 1 to 8,  
30 in which the cells which can be infected by influenza viruses grow adherently.
10. The process as claimed in one of claims 1 to 8, in which the cells which can be infected by influenza viruses grow in suspension.
- 35 11. The process as claimed in one of claims 1 to 10, in which a protease is added to the cultured cells

before, during or after infection with influenza viruses.

12. The process as claimed in claim 11, the protease being a serine protease.

5 13. The process as claimed in claim 12, the serine protease being trypsin.

14. The process as claimed in one of claims 1 to 13, in which the harvesting and isolation of the influenza viruses takes place 2 to 10 days after  
10 infection.

15. The process as claimed in claim 14, in which the harvesting and isolation of the influenza viruses takes place 2 to 7 days after infection.

16. An influenza virus obtainable by a process as  
15 claimed in one of claims 1 to 15.

17. A vaccine containing influenza viruses as claimed in claim 16, if appropriate in combination with substances which increase the immune response.

18. A vaccine as claimed in claim 17, the influenza  
20 viruses being present as intact virus particles.

19. A vaccine as claimed in claim 17, the influenza viruses being present as attenuated viruses.

20. A vaccine as claimed in claim 17, the influenza viruses being present as disintegrated virus particles.

25 21. A vaccine as claimed in claim 21, the vaccine containing isolated proteins of the influenza virus.

22. A diagnostic composition, containing influenza viruses as claimed in claim 16 or constituents of such influenza viruses.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/IB 97/00404

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N5/06 C12N7/00 A61K39/145

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, vol. 397, 1996, pages 141-151, XP002039317 O.-W. MERTEN ET AL.: "PRODUCTION OF INFLUENZA VIRUS IN CELL CULTURES FOR VACCINE PREPARATION" see the whole document ---	1-22
X	REVISTA DE FARMÁCIA E BIOQUÍMICA DA UNIVERSIDADE DE SÃO PAULO, vol. 29, no. 2, July 1993 - December 1993, pages 89-95, XP002039318 DALVA A. PORTARI MANCINI ET AL.: "AVALIAÇÃO DA TRIPSINA NA MULTIPLICAÇÃO DE VÍRUS INFLUENZA EM CULTURAS DE CÉLULAS MDCK" see the whole document ---	1-22
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

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- \*E\* earlier document but published on or after the international filing date
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- \*P\* document published prior to the international filing date but later than the priority date claimed

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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

29 August 1997

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 97/00404

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 1 070 764 A (NORDEN LABORATORIES) 1 June 1967 ---	
A	US 4 500 513 A (BROWN KAREN K ET AL) 19 February 1985 cited in the application -----	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 97/00404

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 1070764 A		BE 665685 A DE 1278075 B FR 1554557 A NL 6508003 A	21-12-65  24-01-69 23-12-65
US 4500513 A	19-02-85	AR 220838 A AU 535804 B AU 5824480 A CA 1122527 A EP 0019218 A JP 1024769 B JP 1540291 C JP 55153723 A US RE33164 E	28-11-80 05-04-84 20-11-80 27-04-82 26-11-80 15-05-89 31-01-90 29-11-80 13-02-90